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(54) **METHODS FOR SUPPRESSING ACUTE REJECTION OF A HEART TRANSPLANT**

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(57) **ABSTRACT**

The effect of anti-IL-6 receptor antibodies in suppressing cytotoxic T cell induction was examined. The results showed that CTL activity against alloantigens was statistically significantly reduced in mice treated with anti-IL-6 receptor antibodies as compared to mice not treated with antibodies and mice treated with a control antibody. The anti-IL-6 receptor antibody was also administered to recipients of a mouse model for allogenic heart transplantation. As a result, histopathological findings showed that inflammatory cell infiltration into transplanted hearts was suppressed and the survival period of transplanted hearts was significantly prolonged. Thus, the present inventors for the first time discovered that administration of anti-IL-6 receptor antibodies could suppress cytotoxic T cell induction and thereby suppress acute rejection after transplantation.

20 Claims, 7 Drawing Sheets

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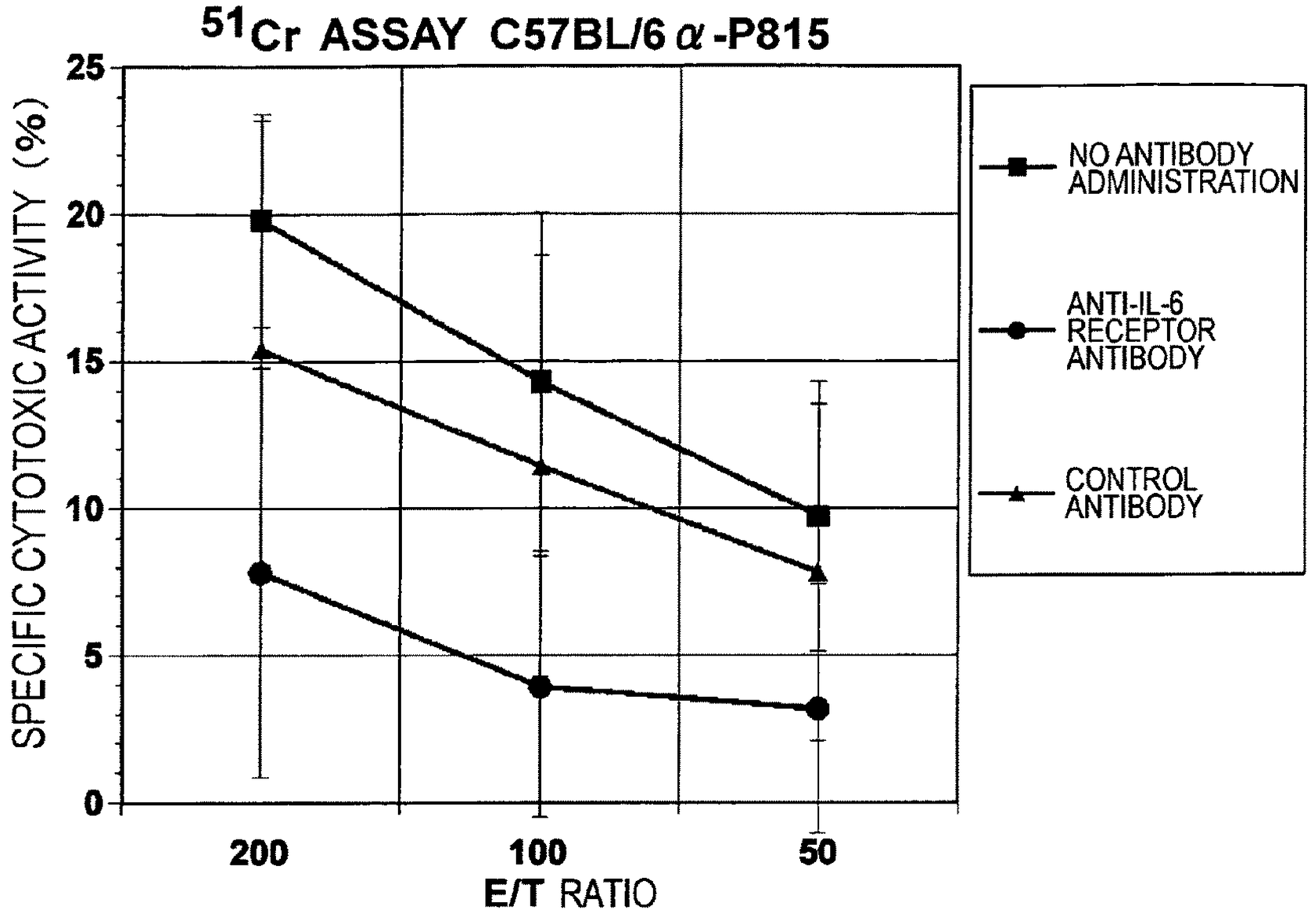
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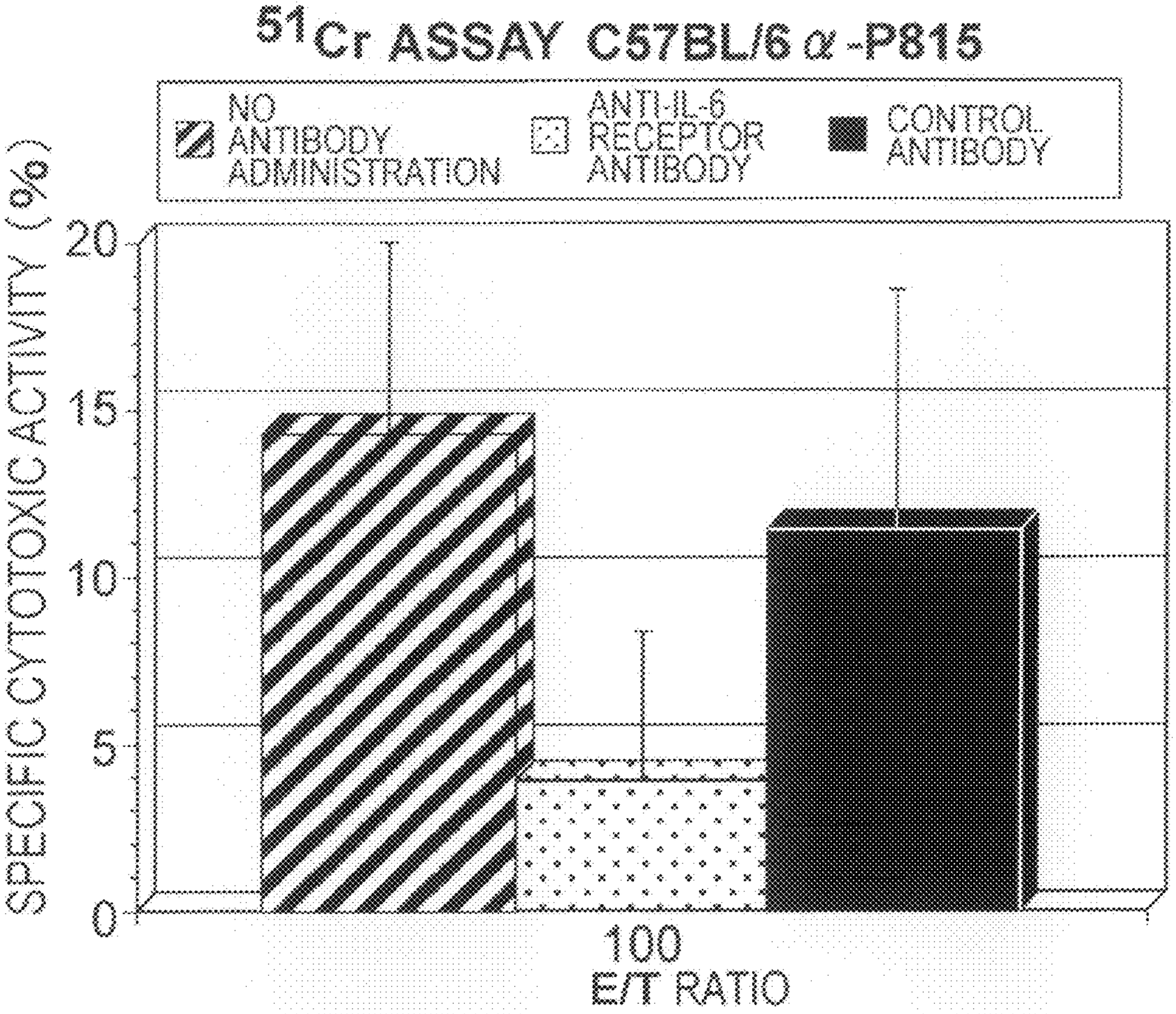
SPECIFIC CYTOTOXIC ACTIVITY (%)
25
20
15
10
5
0
200
100
50
E/T RATIO
NO ANTIBODY ADMINISTRATION
ANTI-IL-6 RECEPTOR ANTIBODY
CONTROL ANTIBODY

FIG. 1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ANTI-IL-6 RECEPTOR ANTIBODY
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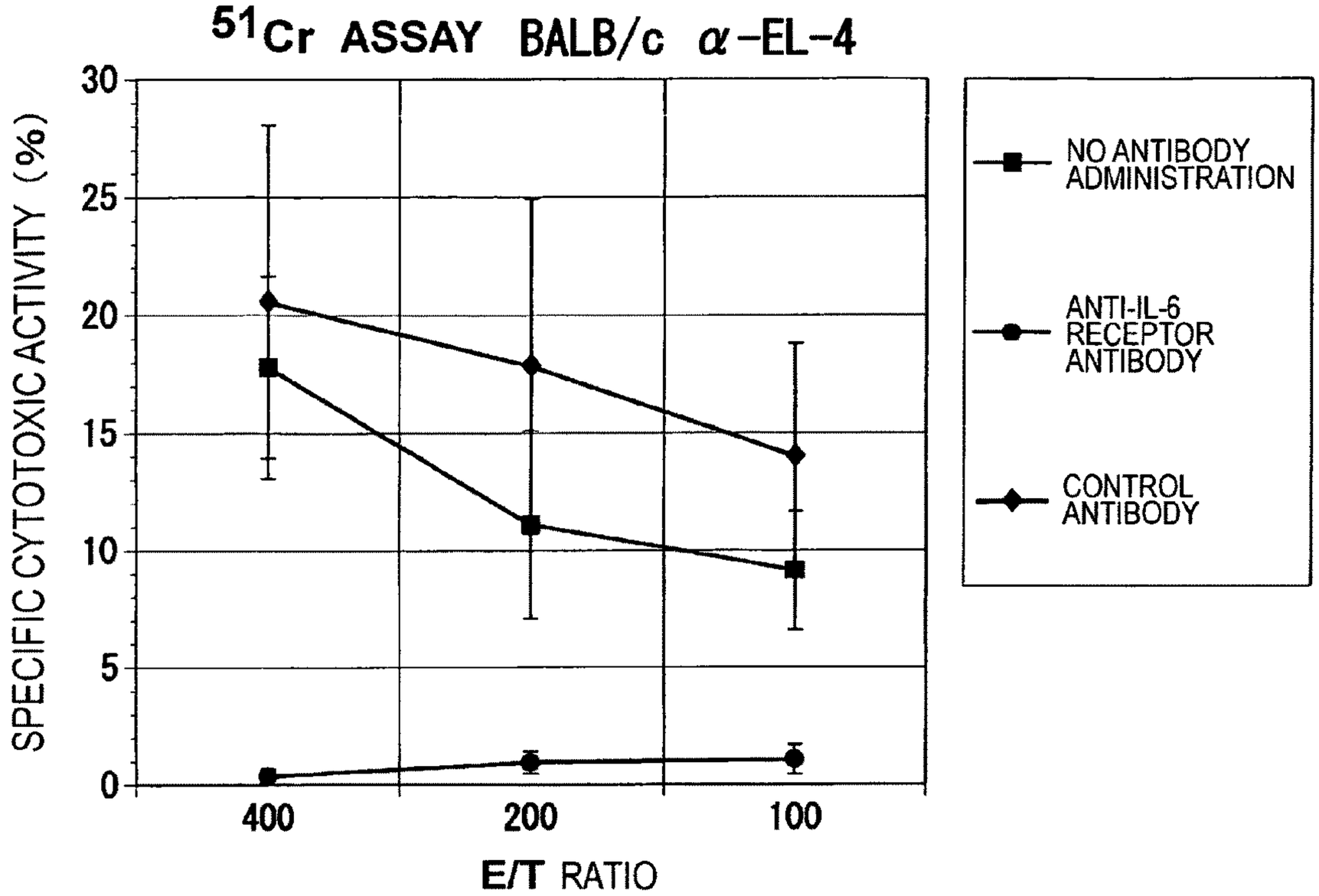
SPECIFIC CYTOTOXIC ACTIVITY (%)
20
15
10
5
0
100
E/T RATIO

FIG. 2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SPECIFIC CYTOTOXIC ACTIVITY (%)
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15
10
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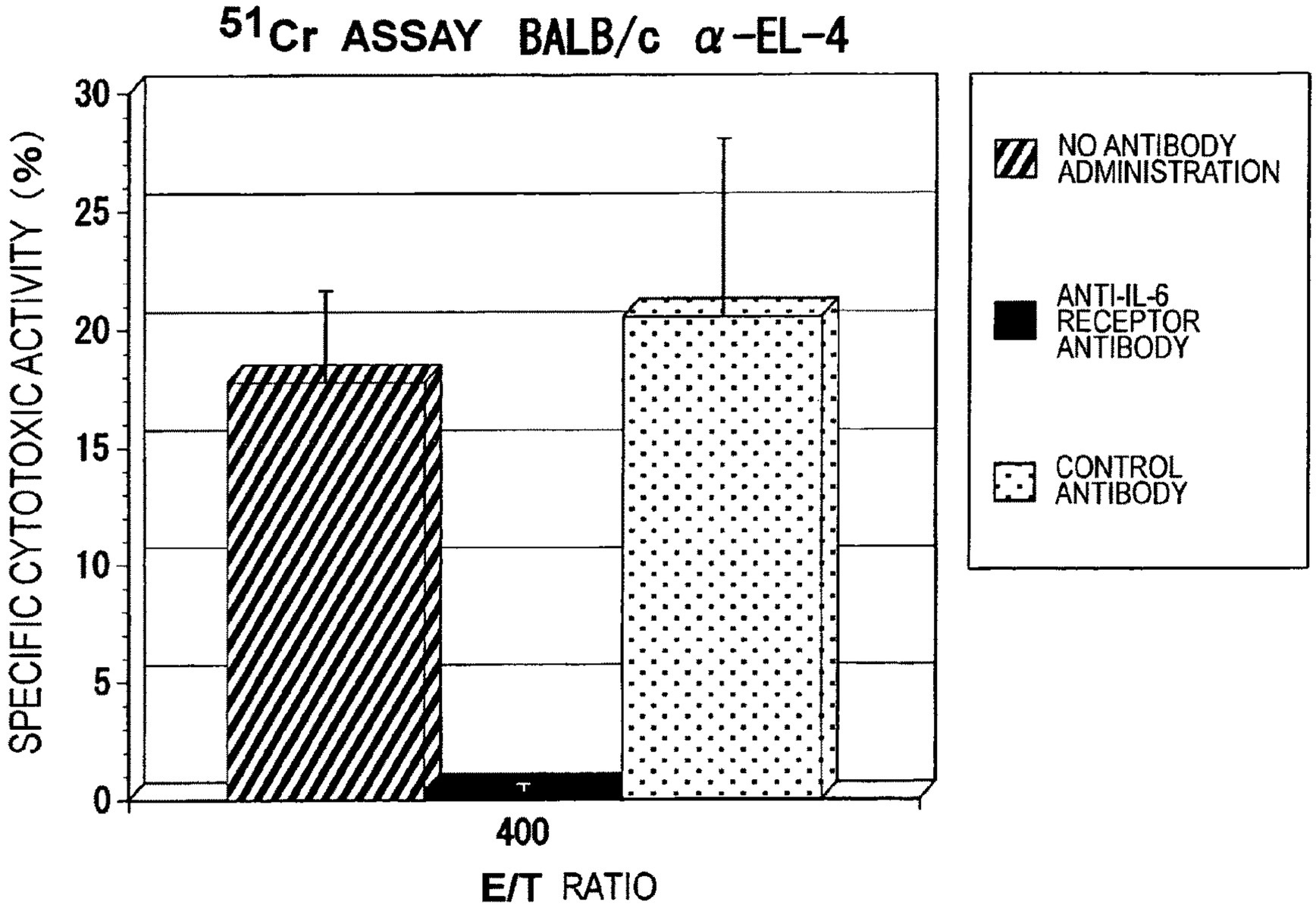
NO ANTIBODY ADMINISTRATION
ANTI-IL-6 RECEPTOR ANTIBODY
CONTROL ANTIBODY

FIG. 3


^{51}Cr ASSAY BALB/c α-EL-4
SPECIFIC CYTOTOXIC ACTIVITY (%)
30
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20
15
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5
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400
E/T RATIO
NO ANTIBODY ADMINISTRATION
ANTI-IL-6 RECEPTOR ANTIBODY
CONTROL ANTIBODY

FIG. 4

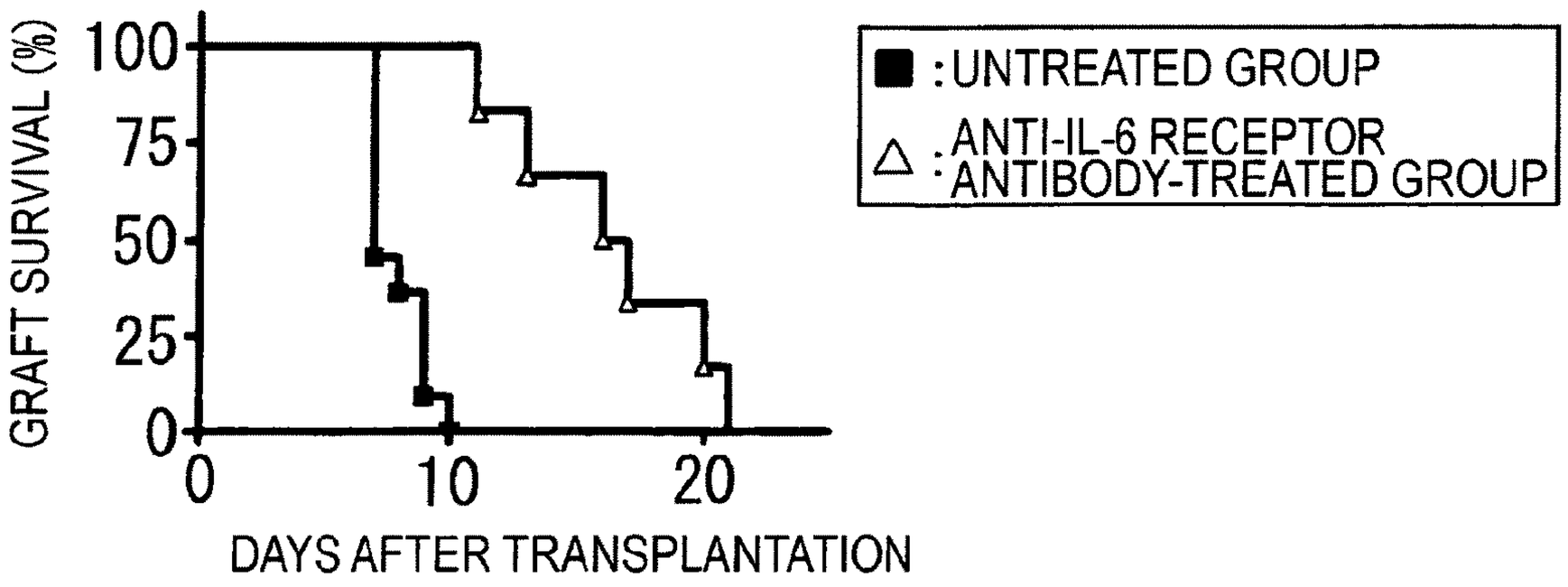
GRAFT SURVIVAL (%)
100
75
50
25
0
0
10
20
DAYS AFTER TRANSPLANTATION
■ : UNTREATED GROUP
△ : ANTI-IL-6 RECEPTOR ANTIBODY-TREATED GROUP

FIG. 5

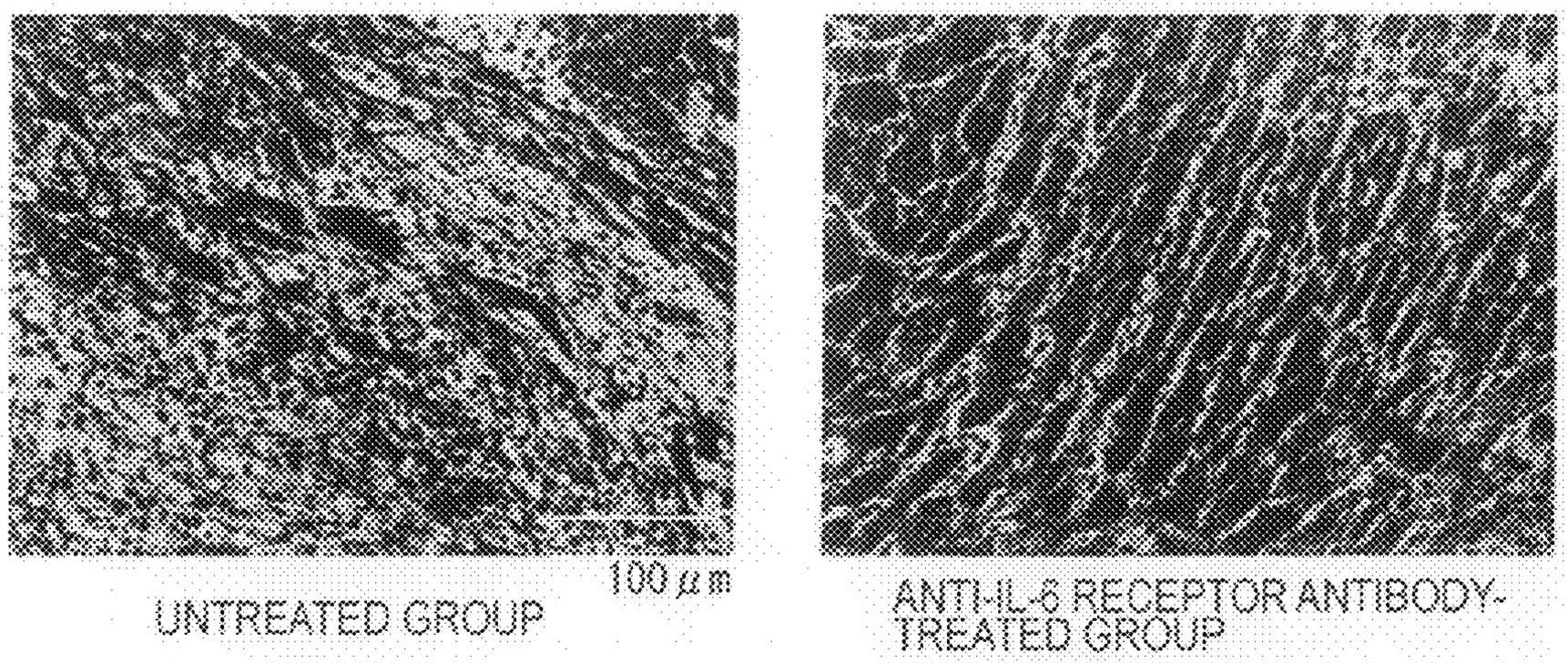
100 μm
UNTREATED GROUP
ANTI-IL-6 RECEPTOR ANTIBODY-TREATED GROUP

FIG. 6

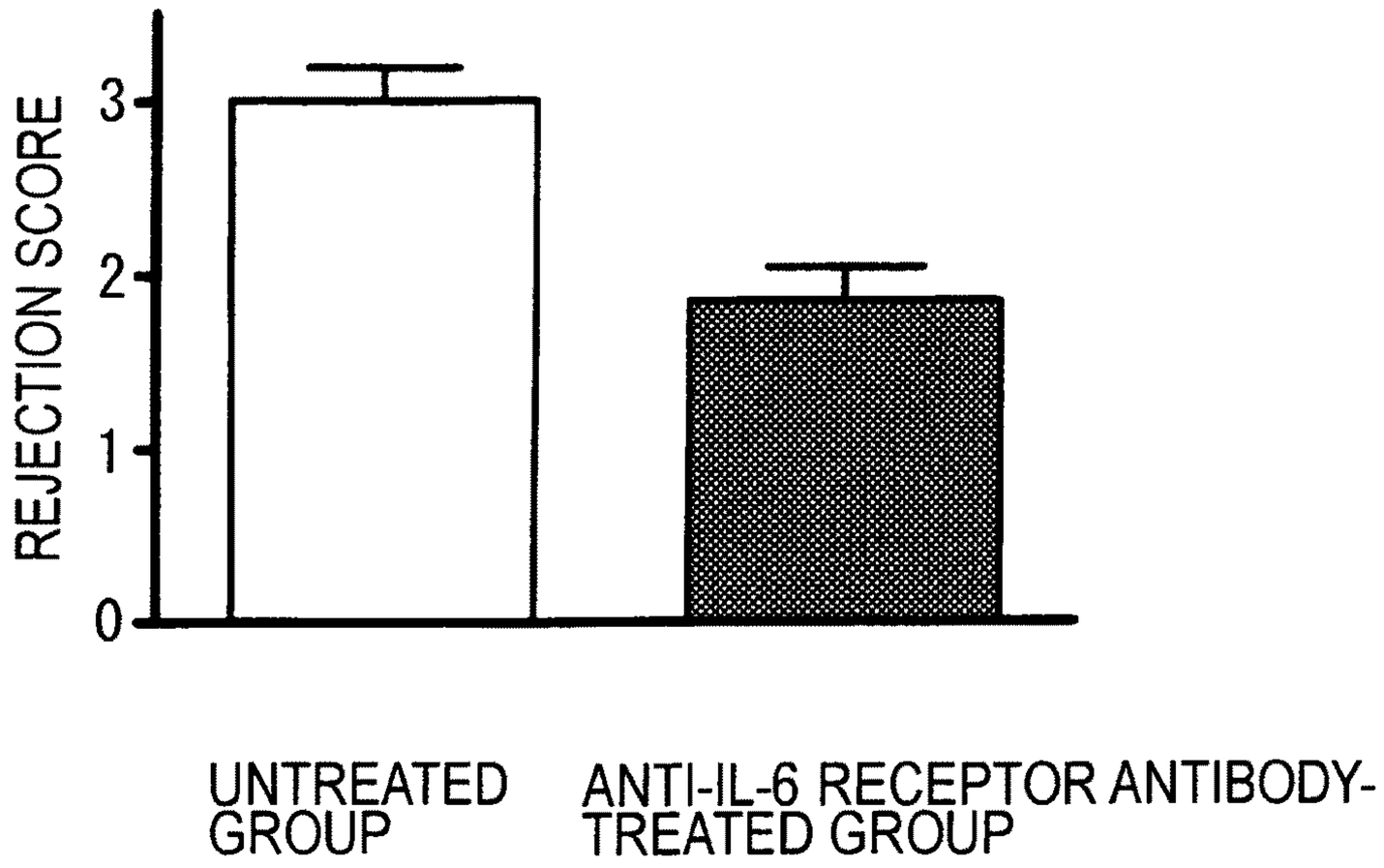
REJECTION SCORE
3
2
1
0
UNTREATED GROUP
ANTI-IL-6 RECEPTOR ANTIBODY-TREATED GROUP

FIG. 7

METHODS FOR SUPPRESSING ACUTE REJECTION OF A HEART TRANSPLANT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is the National Stage of International Application Serial No. PCT/JP2006/322726, filed on Nov. 15, 2006, which claims the benefit of Japanese Application Serial Nos. 2005-330637, filed on Nov. 15, 2005, and 2006-170950, filed on Jun. 21, 2006. The contents of the foregoing applications are hereby incorporated by reference in their entirety.

TECHNICAL FIELD

The present invention relates to agents for suppressing the induction of cytotoxic T cells (killer T cells), which comprise IL-6 inhibitors as active ingredients, and uses thereof. The present invention also relates to methods for suppressing rejections after transplantation, which comprise the step of administering IL-6 inhibitors to recipients.

BACKGROUND ART

IL-6 is a cytokine called B-cell stimulating factor 2 (BSF2) or interferon β2. IL-6 was discovered as a differentiation factor involved in the activation of B-cell lymphocytes (Non-Patent Document 1), and was later revealed to be a multifunctional cytokine that influences the function of various cells (Non-Patent Document 2). IL-6 has been reported to induce maturation of T lymphocyte cells (Non-Patent Document 3).

IL-6 transmits its biological activity via two kinds of proteins on the cell. The first kind of protein is the IL-6 receptor, which is a ligand binding protein to which IL-6 binds; it has a molecular weight of about 80 kDa (Non-Patent Documents 4 and 5). The IL-6 receptor is present in a membrane-bound form that penetrates and is expressed on the cell membrane, and also as a soluble IL-6 receptor, which mainly consists of the extracellular region of the membrane-bound form.

The other kind of protein is the membrane protein gp130, which has a molecular weight of about 130 kDa and is involved in non-ligand binding signal transduction. The biological activity of IL-6 is transmitted into the cell through formation of an IL-6/IL-6 receptor complex by IL-6 and 11-6 receptor followed by binding of the complex with gp130 (Non-Patent Document 6).

IL-6 inhibitors are substances that inhibit the transmission of IL-6 biological activity. Currently, known IL-6 inhibitors include antibodies against IL-6 (anti-IL-6 antibodies), antibodies against IL-6 receptor (anti-IL-6 receptor antibodies), antibodies against gp 130 (anti-gp130 antibodies), IL-6 variants, partial peptides of IL-6 or IL-6 receptor, and such.

There are several reports regarding anti-IL-6 receptor antibodies (Non-Patent Documents 7 and 8; and Patent Documents 1-3). One such report details a humanized PM-1 antibody, which is obtained by transplanting the complementarity determining region (CDR) of mouse antibody PM-1 (Non-Patent Document 9), which is an anti-IL-6 receptor antibody, into a human antibody (Patent Document 4).

IL-6 is known to serve as a killer cell helper factor (KHF) involved in the induction of cytotoxic T cells (Non-Patent Document 10). In-vitro experiments using human rIL-6 show that in the presence of IL-2, rIL-6 induces the differentiation of cytotoxic T cells from human peripheral blood T cells and CD4-/CD8- or CD4-/CD8+ thymus T cells. IL-6 is also known to function in vivo as a cytotoxic T cell differentiation-inducing factor (Non-Patent Document 11).

However, various other cytokines in addition to IL-6 are also known to function as KHFs in vivo, and to date there have been no reports clarifying whether or not IL-6 inhibitors can suppress the induction of cytotoxic T cells in vivo.

The prior-art documents related to the present invention are shown below.

Non-Patent Document 1: Hirano, T. et al., Nature (1986) 324, 73-76
Non-Patent Document 2: Akira, S. et al., Adv. in Immunology (1993) 54, 1-78
Non-Patent Document 3: Lotz, M. et al., J. Exp. Med. (1988) 167, 1253-1258
Non-Patent Document 4: Taga, T. et al., J. Exp. Med. (1987) 166, 967-981
Non-Patent Document 5: Yamasaki, K. et al., Science (1988) 241, 825-828
Non-Patent Document 6: Taga, T. et al., Cell (1989) 58, 573-581
Non-Patent Document 7: Novick, D. et al., Hybridoma (1991) 10, 137-146
Non-Patent Document 8: Huang, Y. W. et al., Hybridoma (1993) 12, 621-630
Non-Patent Document 9: Hirata, Y. et al., J. Immunol. (1989) 143, 2900-2906
Non-Patent Document 10: Okada M. et al., J. Immunology 141:1543-1549, 1988
Non-Patent Document 11: Kitahara M. et al, Jpn. J. Cancer Res. 81:1032-1038.1990
Patent Document 1: WO 95/09873
Patent Document 2: French Patent Application No. FR 2694767
Patent Document 3: U.S. Pat. No. 5,216,128
Patent Document 4: WO 92/19759

DISCLOSURE OF THE INVENTION

Problems to be Solved by the Invention

The present invention has been conducted under the circumstances described above. An objective of the present invention is to provide agents for suppressing the induction of cytotoxic T cells, which comprise IL-6 inhibitors as active ingredients.

A further objective of the present invention is to provide methods for suppressing rejections after transplantation, which comprise the step of administering IL-6 inhibitors to subjects.

Means for Solving the Problems

To achieve the objectives described above, the present inventors tested anti-IL-6 receptor antibodies for the effect of suppressing cytotoxic T cell induction.

First, the present inventors intraperitoneally administered cells of the mastocytoma line P815 into C57BL/6 mice. The inventors also intraperitoneally administered cells of the EL4 lymphoma cell line to BALB/c mice as immunizing cells (alloantigens). Spleens were isolated from these mice, and the splenic cells (effector cells) were cultured at various effector cell/target cell (E/T) ratios, where the above-described immunizing cells were used as target cells, and the CTL activity (cytotoxic T cell activity) per target cell was measured. Then, an anti-IL-6 receptor antibody or rat IgG (control antibody)

was administered to a mouse model having the above-described alloantigens, and CTL activity was assayed under the same conditions.

The result of the above assays showed that CTL activity towards the alloantigens was statistically significantly reduced in mice treated with the anti-IL-6 receptor antibody, as compared with untreated mice and mice treated with the control antibody (FIGS. **1** to **4**). These results clarified that anti-IL-6 receptor antibodies have the function of suppressing cytotoxic T cell induction.

The present inventors also assessed the effect of administering anti-IL-6 receptor antibodies to an allogenic mouse heart transplantation model. As a result, administering anti-IL-6 receptor antibodies suppressed acute heart transplant rejection and statistically significantly prolonged the survival of transplanted hearts. Further, the inventors histopathologically examined transplanted hearts isolated from recipients five days after transplantation. The results showed that in the group treated with anti-IL-6 receptor antibodies, the infiltration of inflammatory cells into transplanted heart tissues was significantly suppressed, and cardiomyocytes were relatively preserved.

Thus, the present inventors discovered for the first time that administering anti-IL-6 receptor antibodies can suppress cytotoxic T cell induction and suppress rejections after transplantation, and they thus completed the present invention.

More specifically, the present invention provides the following inventions:

[1] an agent for suppressing the induction of a cytotoxic T cell, which comprises an IL-6 inhibitor as an active ingredient.

[2] the agent of [1], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6.

[3] the agent of [1], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6 receptor.

[4] the agent of [2] or [3], wherein the antibody is a monoclonal antibody.

[5] the agent of [2] or [3], wherein the antibody recognizes a human IL-6 or a human IL-6 receptor.

[6] the agent of [2] or [3], wherein the antibody is a recombinant antibody.

[7] the agent of [6], wherein the antibody is a chimeric antibody, humanized antibody, or human antibody.

[8] the agent of [1], which is used to suppress rejection after transplantation.

[9] an agent for suppressing rejection in heart transplantation, which comprises an IL-6 inhibitor as an active ingredient.

[10] the agent of [9], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6.

[11] the agent of [9], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6 receptor.

[12] the agent of [10] or [11], wherein the antibody is a monoclonal antibody.

[13] the agent of [10] or [11], wherein the antibody recognizes a human IL-6 or a human IL-6 receptor.

[14] the agent of [10] or [11], wherein the antibody is a recombinant antibody.

[15] the agent of [14], wherein the antibody is a chimeric antibody, humanized antibody, or human antibody.

[16] the agent of [9], which is used to suppress acute rejection in heart transplantation;

[17] a method for suppressing the induction of a cytotoxic T cell, which comprises the step of administering an IL-6 inhibitor to a subject;

[18] the method of [17], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6;

[19] the method of [17], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6 receptor;

[20] the method of [18] or [19], wherein the antibody is a monoclonal antibody;

[21] the method of [18] or [19], wherein the antibody is an antibody that recognizes a human IL-6 or a human IL-6 receptor;

[22] the method of [18] or [19], wherein the antibody is a recombinant antibody;

[23] the method of [22], wherein the antibody is a chimeric antibody, humanized antibody, or human antibody;

[24] the method of [17], which is used to suppress rejection after transplantation;

[25] use of an IL-6 inhibitor in the production of an agent for suppressing the induction of a cytotoxic T cell;

[26] the use of [25], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6;

[27] the use of [25], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6 receptor;

[28] the use of [26] or [27], wherein the antibody is a monoclonal antibody;

[29] the use of [26] or [27], wherein the antibody recognizes a human IL-6 or a human IL-6 receptor;

[30] the use of [26] or [27], wherein the antibody is a recombinant antibody;

[31] the use of [30], wherein the antibody is a chimeric antibody, humanized antibody, or human antibody;

[32] a method for suppressing rejection in heart transplantation, which comprises the step of administering an IL-6 inhibitor to a subject;

[33] the method of [32], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6;

[34] the method of [32], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6 receptor;

[35] the method of [33] or [34], wherein the antibody is a monoclonal antibody;

[36] the method of [33] or [34], wherein the antibody recognizes a human IL-6 or a human IL-6 receptor;

[37] the method of [33] or [34], wherein the antibody is a recombinant antibody;

[38] the method of [37], wherein the antibody is a chimeric antibody, humanized antibody, or human antibody;

[39] the method of [32], which is used to suppress acute rejection in heart transplantation;

[40] use of an IL-6 inhibitor in the production of an agent for suppressing rejection in heart transplantation;

[41] the use of [40], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6;

[42] the use of [40], wherein the IL-6 inhibitor is an antibody that recognizes an IL-6 receptor;

[43] the use of [41] or [42], wherein the antibody is a monoclonal antibody;

[44] the use of [41] or [42], wherein the antibody recognizes a human IL-6 or a human IL-6 receptor;

[45] the use of [41] or [42], wherein the antibody is a recombinant antibody; and

[46] the use of [45], wherein the antibody is a chimeric antibody, humanized antibody, or human antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the specific cytotoxic activity (cytotoxic T lymphocyte activity; CTL activity) for each E/T ratio in mice not treated with an antibody, mice treated with an anti-IL-6 receptor antibody, and mice treated with a control antibody, where each was administered with cells of the mastocytoma line P815.

FIG. **2** shows the specific cytotoxic activity (CTL activity) when the E/T ratio is 100 in mice not treated with an antibody, mice treated with an anti-IL-6 receptor antibody, and mice treated with a control antibody, where each was administered with cells of mastocytoma line P815.

FIG. **3** shows the specific cytotoxic activity (CTL activity) at each E/T ratio in mice not treated with an antibody, mice treated with an anti-IL-6 receptor antibody, and mice treated with a control antibody, where each was administered with EL4 lymphoma cells.

FIG. **4** shows the specific cytotoxic activity (CTL activity) when the E/T ratio is 400 in mice not treated with an antibody, mice treated with an anti-IL-6 receptor antibody, and mice treated with a control antibody, where each was administered with EL4 lymphoma cells.

FIG. **5** shows the viability of transplanted hearts in the group treated with an anti-IL-6 receptor antibody and the untreated group, in the mouse model for heart transplantation.

FIG. **6** is photographs showing histopathological images of transplanted hearts five days after transplantation, for the group treated with an anti-IL-6 receptor antibody and the untreated group, in the mouse model for heart transplantation.

FIG. **7** shows the results of comparing rejection scores for transplanted hearts five days after transplantation between the group treated with an anti-IL-6 receptor antibody and the untreated group, in the mouse model for heart transplantation.

BEST MODE FOR CARRYING OUT THE INVENTION

The present inventors discovered that administration of an anti-IL-6 receptor antibody can suppress cytotoxic T cell induction. The present invention is based on these findings.

The present invention relates to agents for suppressing cytotoxic T cell induction, which comprise an IL-6 inhibitor as an active ingredient.

Herein, an "IL-6 inhibitor" is a substance that blocks IL-6-mediated signal transduction and inhibits IL-6 biological activity. Preferably, the IL-6 inhibitor is a substance that has inhibitory function against the binding of IL-6, IL-6 receptor, or gp130.

The IL-6 inhibitors of the present invention include, but are not limited to, for example, anti-IL-6 antibodies, anti-IL-6 receptor antibodies, anti-gp130 antibodies, IL-6 variants, soluble IL-6 receptor variants, and partial peptides of IL-6 or IL-6 receptor and low molecular weight compounds that show similar activities. Preferable IL-6 inhibitors of the present invention include antibodies that recognize IL-6 receptors.

The source of the antibodies is not particularly restricted in the present invention; however, the antibodies are preferably derived from mammals, and more preferably derived from humans.

The anti-IL-6 antibodies used in the present invention can be obtained as polyclonal or monoclonal antibodies using known means. In particular, monoclonal antibodies derived from mammals are preferred as the anti-IL-6 antibodies used in the present invention. Monoclonal antibodies derived from mammals include those produced from hybridomas and those produced by genetic engineering methods from hosts transformed with an expression vector that comprises an antibody gene. By binding to IL-6, the antibody inhibits IL-6 from binding to an IL-6 receptor and thus blocks the transmission of IL-6 biological activity into the cell.

Such antibodies include, MH166 (Matsuda, T. et al., Eur. J. Immunol. (1988) 18, 951-956), SK2 antibody (Sato, K. et al., transaction of the 21st Annual Meeting of the Japanese Society for Immunology (1991) 21, 166), and so on.

Basically, hybridomas that produce anti-IL-6 antibodies can be prepared using known techniques, as follows: Specifically, such hybridomas can be prepared by using IL-6 as a sensitizing antigen to carry out immunization using a conventional immunization method, fusing the obtained immune cells with known parent cells by a conventional cell fusion method, and screening for monoclonal antibody-producing cells using a conventional screening method.

More specifically, anti-IL-6 antibodies can be produced as follows: For example, human IL-6 for use as the sensitizing antigen for obtaining antibodies can be obtained using the IL-6 gene and/or amino acid sequences disclosed in Eur. J. Biochem. (1987) 168, 543-550; J. Immunol. (1988) 140, 1534-1541; and/or Agr. Biol. Chem. (1990) 54, 2685-2688.

After transforming an appropriate host cell with a known expression vector system inserted with an IL-6 gene sequence, the desired IL-6 protein is purified using known methods from the inside of the host cell or from the culture supernatant. This purified IL-6 protein may be used as a sensitizing antigen. Alternatively, a fusion protein of the IL-6 protein and another protein may be used as a sensitizing antigen.

Anti-IL6 receptor antibodies used for the present invention can be obtained as polyclonal or monoclonal antibodies by using known methods. In particular, the anti-IL-6 receptor antibodies used in the present invention are preferably monoclonal antibodies derived from mammals. The monoclonal antibodies derived from mammals include those produced from hybridomas and those produced using genetic engineering methods from hosts transformed with an expression vector that comprises an antibody gene. By binding to an IL-6 receptor, the antibody inhibits IL-6 from binding to the IL-6 receptor, and thus blocks the transmission of IL-6 biological activity into the cell.

Such antibodies include, MR16-1 antibody (Tamura, T. et al., Proc. Natl. Acad. Sci. USA (1993) 90, 11924-11928); PM-1 antibody (Hirata, Y. et al., J. Immunol. (1989) 143, 2900-2906); AUK12-20 antibody, AUK64-7 antibody and AUK146-15 antibody (WO 92/19759); and so on. Of these, the PM-1 antibody can be exemplified as a preferred monoclonal antibody against the human IL-6 receptor, and the MR16-1 antibody as a preferred monoclonal antibody against the mouse IL-6 receptor.

Basically, hybridomas producing an anti-IL-6 receptor monoclonal antibody can be prepared using known techniques, as follows: Specifically, such hybridomas can be prepared by using an IL-6 receptor as the sensitizing antigen to carry out immunization by a conventional immunization method, fusing the obtained immune cells with a known parent cell using a conventional cell fusion method, and screening for monoclonal antibody-producing cells using a conventional screening method.

More specifically, anti-IL-6 receptor antibodies can be produced as follows: For example, a human IL-6 receptor or mouse IL-6 receptor for use as a sensitizing antigen for obtaining antibodies can be obtained by using the IL-6 receptor genes and/or amino acid sequences disclosed in European Patent Application Publication No. EP 325474 and Japanese Patent Application Kokai Publication No. (JP-A) Hei 3-155795, respectively.

There are two kinds of IL-6 receptor proteins: one expressed on the cell membrane and the other separated from the cell membrane (soluble IL-6 receptors) (Yasukawa, K. et al., J. Biochem. (1990) 108, 673-676). The soluble IL-6 receptor essentially consists of the extracellular region of the

cell membrane-bound IL-6 receptor, and differ from the membrane-bound IL-6 receptor in that it lacks the transmembrane region or both the transmembrane and intracellular regions. Any IL-6 receptor may be employed as an IL-6 receptor protein, so long as it can be used as a sensitizing antigen for producing an anti-IL-6 receptor antibody used in the present invention.

After transforming an appropriate host cell with a known expression vector system inserted with an IL-6 receptor gene sequence, the desired IL-6 receptor protein is purified from the inside of the host cell or from the culture supernatant using a known method. This purified IL-6 receptor protein may be used as a sensitizing antigen. Alternatively, a cell expressing the IL-6 receptor or a fusion protein of the IL-6 receptor protein and another protein may be used as a sensitizing antigen.

Anti-gp130 antibodies used in the present invention can be obtained as polyclonal or monoclonal antibodies by using known methods. In particular, the anti-gp130 antibodies used in the present invention are preferably monoclonal antibodies derived from mammals. Mammal-derived monoclonal antibodies include those produced from hybridomas and those produced using genetic engineering methods from hosts transformed with an expression vector that comprises an antibody gene. By binding to gp130, the antibody inhibits gp130 from binding to the IL-6/IL-6 receptor complex, and thus blocks transmission of IL-6 biological activity into the cell.

Such antibodies include, AM64 antibody (JP-A Hei 3-219894); 4B11 antibody and 2H4 antibody (U.S. Pat. No. 5,571,513); B-S12 antibody and B-P8 antibody (JP-A Hei 8-291199); and so on.

Basically, anti-gp130 monoclonal antibody-producing hybridomas can be prepared using known techniques, as follows: Specifically, such hybridomas can be prepared by using gp130 as a sensitizing antigen to carry out the immunization using a conventional immunization method, fusing the obtained immune cells with a known parent cell by a conventional cell fusion method, and screening for monoclonal antibody-producing cells using a conventional screening method.

More specifically, monoclonal antibodies can be produced as follows: For example, gp130 for use as a sensitizing antigen for obtaining antibodies can be obtained using the gp130 gene and/or amino acid sequence disclosed in European Patent Application Publication No. EP 411946.

After transforming an appropriate host cell with a known expression vector system inserted with a gp130 gene sequence, the desired gp130 protein is purified by a known method from the inside of the host cell or from the culture supernatant. This purified gp130 protein may be used as a sensitizing antigen. Alternatively, a cell expressing gp130 or a fusion protein of the gp130 protein and another protein may be used as a sensitizing antigen.

Mammals to be immunized with a sensitizing antigen are not particularly limited, but are preferably selected in consideration of compatibility with the parent cell used for cell fusion. Generally, rodents such as mice, rats, and hamsters are used.

Animals are immunized with sensitizing antigens according to known methods. For example, as a general method, animals are immunized by intraperitoneal or subcutaneous injection of a sensitizing antigen. Specifically, the sensitizing antigen is preferably diluted or suspended in an appropriate amount of phosphate-buffered saline (PBS), physiological saline or such, mixed with an appropriate amount of a general adjuvant (e.g., Freund's complete adjuvant), emulsified, and then administered to a mammal several times, every four to 21 days. In addition, an appropriate carrier may be used for immunization with a sensitizing antigen.

Following such immunization, an increased level of a desired antibody in serum is confirmed and then immune cells are obtained from the mammal for cell fusion. Preferred immune cells for cell fusion include, in particular, spleen cells.

The mammalian myeloma cells used as parent cells, i.e. as partner cells to be fused with the above immune cells, include various known cell strains, for example, P3X63Ag8.653 (Kearney, J. F. et al., J. Immunol (1979) 123, 1548-1550), P3X63Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81, 1-7), NS-1 (Kohler, G and Milstein, C., Eur. J. Immunol. (1976) 6, 511-519), MPC-11 (Margulies, D. H. et al., Cell (1976) 8, 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270), F0 (de St. Groth, S. F. et al., J. Immunol. Methods (1980) 35, 1-21), S194 (Trowbridge, I. S., J. Exp. Med. (1978) 148, 313-323), R210 (Galfre, G et al., Nature (1979) 277, 131-133), and such.

Basically, cell fusion of the aforementioned immune cells and myeloma cells can be performed using known methods, for example, the method of Milstein et al. (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46), and such.

More specifically, the aforementioned cell fusion is achieved in general nutrient culture medium in the presence of a cell fusion enhancing agent. For example, polyethylene glycol (PEG), Sendai virus (HVJ), and such are used as fusion enhancing agents. Further, to enhance fusion efficiency, auxiliary agents such as dimethyl sulfoxide may be added depending on needs.

The ratio of immune cells to myeloma cells used is preferably, for example, 1 to 10 immune cells for each myeloma cell. The culture medium used for the aforementioned cell fusion is, for example, the RPMI 1640 or MEM culture medium, which are suitable for proliferation of the aforementioned myeloma cells. A general culture medium used for culturing this type of cell can also be used. Furthermore, serum supplements such as fetal calf serum (FCS) can be used in combination.

For cell fusion, the fusion cells (hybridomas) of interest are formed by mixing predetermined amounts of an aforementioned immune cell and myeloma cell in an aforementioned culture medium, and then adding and mixing a concentration of 30% to 60% (w/v) PEG solution (e.g., a PEG solution with a mean molecular weight of about 1,000 to 6,000) pre-heated to about 37° C. Then, cell fusion agents and such that are unsuitable for the growth of hybridomas can be removed by repeatedly adding an appropriate culture medium and then removing the supernatant by centrifugation.

The above hybridomas are selected by culturing cells in a general selection culture medium, for example, HAT culture medium (a culture medium containing hypoxanthine, aminopterin, and thymidine). Culture in HAT culture medium is continued for a sufficient period, generally several days to several weeks, to kill cells other than the hybridomas of interest (unfused cells). Then, a standard limited dilution method is performed to screen and clone hybridomas that produce an antibody of interest.

In addition to the methods for immunizing non-human animals with antigens for obtaining the aforementioned hybridomas, desired human antibodies with the activity of binding to a desired antigen or antigen-expressing cell can be obtained by sensitizing a human lymphocyte with a desired antigen protein or antigen-expressing cell in vitro, and fusing the sensitized B lymphocyte with a human myeloma cell (e.g., U266) (see, Japanese Patent Application Kokoku Publication No. (JP-B) Hei 1-59878 (examined, approved Japa-

nese patent application published for opposition)). Further, a desired human antibody can be obtained by administering an antigen or antigen-expressing cell to a transgenic animal that has a repertoire of human antibody genes, and then following the aforementioned method (see, International Patent Application Publication Nos. WO 93/12227, WO 92/03918, WO 94/02602, WO 94/25585, WO 96/34096, and WO 96/33735).

The thus-prepared hybridomas which produce monoclonal antibodies can be subcultured in a conventional culture medium and stored in liquid nitrogen for a long period.

When obtaining monoclonal antibodies from the aforementioned hybridomas, the following methods may be employed: (1) methods where the hybridomas are cultured according to conventional methods and the antibodies are obtained as a culture supernatant; (2) methods where the hybridomas are proliferated by administering them to a compatible mammal and the antibodies are obtained as ascites; and so on. The former method is preferred for obtaining antibodies with high purity, and the latter is preferred for large-scale antibody production.

For example, anti-IL-6 receptor antibody-producing hybridomas can be prepared by the method disclosed in JP-A Hei 3-139293. Such hybridomas can be prepared by injecting a PM-1 antibody-producing hybridoma into the abdominal cavity of a BALB/c mouse, obtaining ascites, and then purifying a PM-1 antibody from the ascites; or by culturing the hybridoma in an appropriate medium (e.g., RPMI1640 medium containing 10% fetal bovine serum, and 5% BM-Condimed H1 (Boehringer Mannheim); hybridoma SFM medium (GIBCO-BRL); PFHM-II medium (GIBCO-BRL), etc.) and then obtaining PM-1 antibody from the culture supernatant.

Recombinant antibodies can be used as the monoclonal antibodies of the present invention, wherein the antibodies are produced using genetic recombination techniques by cloning an antibody gene from a hybridoma, inserting the gene into an appropriate vector, and then introducing the vector into a host (see, for example, Borrebaeck, C. A. K. and Larrick, J. W., Therapeutic Monoclonal Antibodies, published in the United Kingdom by Macmillan Publishers Ltd, 1990).

More specifically, mRNAs coding for antibody variable (V) regions are isolated from cells that produce antibodies of interest, such as hybridomas. mRNAs can be isolated by preparing total RNAs according to known methods, such as the guanidine ultracentrifugation method (Chirgwin, J. M. et al., Biochemistry (1979) 18, 5294-5299) and the AGPC method (Chomczynski, P. et al., Anal. Biochem. (1987) 162, 156-159), and preparing mRNAs using the an MRNA Purification Kit (Pharmacia) and such. Alternatively, mRNAs can be directly prepared using a QuickPrep MRNA Purification Kit (Pharmacia).

cDNAs of the antibody V regions are synthesized from the obtained mRNAs using reverse transcriptase. cDNAs may be synthesized using an AMV Reverse Transcriptase First-strand cDNA Synthesis Kit and so on. Further, to synthesize and amplify the cDNAs, the 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002; Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932) using 5'-Ampli FINDER RACE Kit (Clontech) and PCR may be employed. A DNA fragment of interest is purified from the obtained PCR products and then ligated with a vector DNA. Then, a recombinant vector is prepared using the above DNA and introduced into *Escherichia coli* or such, and then its colonies are selected to prepare a desired recombinant vector. The nucleotide sequence of the DNA of interest is confirmed by, for example, the dideoxy method.

When a DNA encoding the V region of an antibody of interest is obtained, the DNA is ligated with a DNA that encodes a desired antibody constant region (C region), and inserted into an expression vector. Alternatively, a DNA encoding an antibody V region may be inserted into an expression vector comprising a DNA of an antibody C region.

To produce an antibody to be used in the present invention, as described below, an antibody gene is inserted into an expression vector such that it is expressed under the control of an expression regulating region, for example, an enhancer and promoter. Then, the antibody can be expressed by transforming a host cell with this expression vector.

In the present invention, to reduce heteroantigenicity against humans and such, artificially modified genetic recombinant antibodies, for example, chimeric antibodies, humanized antibodies, or human antibodies, can be used. These modified antibodies can be prepared using known methods.

A chimeric antibody can be obtained by ligating a DNA encoding an antibody V region, obtained as above, with a DNA encoding a human antibody C region, then inserting the DNA into an expression vector and introducing it into a host for production (see, European Patent Application Publication No. EP 125023; International Patent Application Publication No. WO 92/19759). This known method can be used to obtain chimeric antibodies useful for the present invention.

Humanized antibodies are also referred to as reshaped human antibodies, and are antibodies wherein the complementarity determining regions (CDRs) of an antibody from a mammal other than human (e.g., a mouse antibody) are transferred into the CDRs of human antibodies. General methods for this gene recombination are also known (see, European Patent Application Publication No. EP 125023, International Patent Application Publication No. WO 92/19759).

More specifically, DNA sequences designed such that the CDRs of a mouse antibody are ligated with the framework regions (FRs) of a human antibody are synthesized by PCR from several oligonucleotides produced to contain overlapping portions at their termini. The obtained DNA is ligated with a human antibody C region-encoding DNA and then inserted into an expression vector. The expression vector is introduced into a host to produce the humanized antibody (see, European Patent Application Publication No. EP 239400, International Patent Application Publication No. WO 92/19759).

The human antibody FRs to be ligated via the CDRs are selected so that the CDRs form suitable antigen binding sites. The amino acid(s) within the FRs of the antibody variable regions may be substituted as necessary so that the CDRs of the reshaped human antibody form an appropriate antigen binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

Human antibody C regions are used for the chimeric and humanized antibodies, and include Cγ. For example, Cγ1, Cγ2, Cγ3, or Cγ4 may be used. Furthermore, to improve the stability of the antibodies or their production, the human antibody C regions may be modified.

Chimeric antibodies consist of the variable region of an antibody derived from a non-human mammal and the constant region of an antibody derived from a human; humanized antibodies consist of the CDRs of an antibody derived from a non-human mammal and the framework regions and constant regions derived from a human antibody. Both have reduced antigenicity in the human body, and are thus useful as antibodies for use in the present invention.

Preferred specific examples of humanized antibodies for use in the present invention include the humanized PM-1 antibody (see, International Patent Application Publication No. WO 92/19759).

Furthermore, in addition to the aforementioned methods for obtaining human antibodies, techniques for obtaining human antibodies by panning using a human antibody library are also known. For example, the variable regions of human antibodies can be expressed on phage surfaces as single chain antibodies (scFv) by using the phage display method, and antigen-binding phages can then be selected. By analyzing the genes of the selected phages, DNA sequences coding for the human antibody variable regions that bind to the antigen can be determined. Once the DNA sequence of an scFv that binds to the antigen is revealed, an appropriate expression vector comprising the sequence can be constructed to obtain an human antibody. These methods are already known, and the publications of WO 92/01047, WO 92/20791, WO93/06213, WO 93/11236, WO 93/19172, WO 95/01438, and WO 95/15388 can be used as reference.

The antibody genes constructed above can be expressed according to conventional methods. When a mammalian cell is used, the antibody gene can be expressed using a DNA in which the antibody gene to be expressed is functionally ligated to a useful commonly used promoter and a poly A signal downstream of the antibody gene, or a vector comprising the DNA. Examples of a promoter/enhancer include the human cytomegalovirus immediate early promoter/enhancer.

Furthermore, other promoters/enhancers that can be used for expressing the antibodies for use in the present invention include viral promoters/enhancers from retroviruses, polyoma viruses, adenoviruses, simian virus 40 (SV40), and such; and also include mammalian cell-derived promoters/enhancers such as human elongation factor 1α (HEF 1α).

For example, when the SV40 promoter/enhancer is used, the expression can be easily performed by following the method by Mulligan et al. (Mulligan, R. C. et al., Nature (1979) 277, 108-114). Alternatively, in the case of the HEF1α promoter/enhancer, the method by Mizushima et al. (Mizushima, S. and Nagata S., Nucleic Acids Res. (1990) 18, 5322) can be used.

When *E. coli* is used, an antibody gene can be expressed by functionally ligating a conventional promoter, a signal sequence for antibody secretion, and the antibody gene to be expressed. Examples of the promoter include a lacZ promoter, araB promoter and such. When a lacZ promoter is used, genes can be expressed according to the method of Ward et al. (Ward, E. S. et al., Nature (1989) 341, 544-546; Ward, E. S. et al., FASEB J. (1992) 6, 2422-2427); and the araB promoter may be used according to the method of Better et al. (Better, M. et al., Science (1988) 240, 1041-1043).

When the antibody is produced into the periplasm of *E. coli*, the pel B signal sequence (Lei, S. P. et al., J. Bacteriol. (1987) 169, 4379-4383) may be used as a signal sequence for antibody secretion. The antibodies produced into the periplasm are isolated, and then used after appropriately refolding the antibody structure (see, for example, WO 96/30394).

As the replication origin, those derived from SV40, polyoma virus, adenovirus, bovine papilloma virus (BPV) and such may be used. In addition, to enhance the gene copy number in a host cell system, the expression vector may comprise the aminoglycoside phosphotransferase (APH) gene, thymidine kinase (TK) gene, *E. coli* xanthine-guanine phosphoribosyltransferase (Ecogpt) gene, dihydrofolate reductase (dhfr) gene, or such as a selection marker.

Any production system may be used to prepare the antibodies for use in the present invention. The production systems for antibody preparation include in vitro and in vivo production systems. In vitro production systems include those using eukaryotic cells or prokaryotic cells.

Production systems using eukaryotic cells include those using animal cells, plant cells, or fungal cells. Such animal cells include (1) Mammalian cells, for example, CHO, COS, myeloma, baby hamster kidney (BHK), HeLa, Vero, and such; (2) amphibian cells, for example, *Xenopus* oocyte; and (3) insect cells, for example, sf9, sf21, Tn5, and such. Known plant cells include cells derived from *Nicotiana tabacum*, which may be cultured as a callus. Known fungal cells include yeasts such as *Saccharomyces* (e.g., *S. cerevisiae*), mold fungi such as *Aspergillus* (e.g., *A. niger*), and such.

Production systems using prokaryotic cells include those using bacterial cells. Known bacterial cells include *E. coli* and *Bacillus subtilis*.

Antibodies can be obtained by using transformation to introduce an antibody gene of interest into these cells, and then culturing the transformed cells in vitro. Cultures are conducted according to known methods. For example, DMEM, MEM, RPMI1640, IMDM may be used as the culture medium, and serum supplements such as FCS may be used in combination. Further, cells introduced with antibody genes may be transferred into the abdominal cavity or such of an animal to produce the antibodies in vivo.

On the other hand, in vivo production systems include those using animals or plants. Production systems using animals include those that use mammals or insects.

Mammals that can be used include goats, pigs, sheep, mice, bovines and such (Vicki Glaser, SPECTRUM Biotechnology Applications, 1993). Further, insects that can be used include silkworms. When using plants, tobacco may be used, for example.

An antibody gene is introduced into these animals or plants, the antibody is produced in the body of the animals or plants, and this antibody is then recovered. For example, an antibody gene can be prepared as a fusion gene by inserting it into the middle of a gene encoding a protein such as goat β casein, which is uniquely produced into milk. DNA fragments comprising the fusion gene, which includes the antibody gene, are injected into goat embryos, and the embryos are introduced into female goats. The desired antibody is obtained from milk produced by the transgenic animals born to the goats that received the embryos, or produced from progenies of these animals. The transgenic goats can be given hormones to increase the volume of milk containing the desired antibody that they produce (Ebert, K. M. et al., Bio/Technology (1994) 12, 699-702).

When silkworms are used, the silkworms are infected with a baculovirus inserted with a desired antibody gene, and the desired antibody is obtained from the body fluids of these silkworm (Maeda, S. et al., Nature (1985) 315, 592-594). Moreover, when tobacco is used, the desired antibody gene is inserted into a plant expression vector (e.g., pMON530) and the vector is introduced into bacteria such as *Agrobacterium tumefaciens*. This bacterium is used to infect tobacco (e.g., *Nicotiana tabacum*) such that desired antibodies can be obtained from the leaves of this tobacco (Julian, K.-C. Ma et al., Eur. J. Immunol. (1994) 24, 131-138).

When producing antibodies using in vitro or in vivo production systems, as described above, DNAs encoding an antibody heavy chain (H chain) and light chain (L chain) may be inserted into separate expression vectors and a host is then co-transformed with the vectors. Alternatively, the DNAs may be inserted into a single expression vector for transforming a host (see International Patent Application Publication No. WO 94/11523).

The antibodies used in the present invention may be antibody fragments or modified products thereof, so long as they can be suitably used in the present invention. For example,

antibody fragments include Fab, F(ab')2, Fv, and single chain Fv (scFv), in which the Fvs of the H and L chains are linked via an appropriate linker.

Specifically, the antibody fragments are produced by treating antibodies with enzymes, for example, papain or pepsin, or alternatively, genes encoding these fragments are constructed, introduced into expression vectors, and these are expressed in appropriate host cells (see, for example, Co, M. S. et al., J. Immunol. (1994) 152, 2968-2976; Better, M. & Horwitz, A. H., Methods in Enzymology (1989) 178, 476-496; Plueckthun, A. & Skerra, A., Methods in Enzymology (1989) 178, 497-515; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. et al., Methods in Enzymology (1989) 121, 663-666; Bird, R. E. et al., TIBTECH (1991) 9, 132-137).

An scFv can be obtained by linking the H-chain V region and the L-chain V region of an antibody. In the scFv, the H-chain V region and the L-chain V region are linked via a linker, preferably via a peptide linker (Huston, J. S. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 5879-5883). The V regions of the H and L chains in an scFv may be derived from any of the antibodies described above. Peptide linkers for linking the V regions include, for example, arbitrary single chain peptides consisting of 12 to 19 amino acid residues.

An scFv-encoding DNA can be obtained by using a DNA encoding an H chain or a V region and a DNA encoding an L chain or a V region of the aforementioned antibodies as templates, using PCR to amplify a DNA portion that encodes the desired amino acid sequence in the template sequence and uses primers that define the termini of the portion, and then further amplifying the amplified DNA portion with a DNA that encodes a peptide linker portion and primer pairs that link both ends of the linker to the H chain and L chain.

Once an scFv-encoding DNA has been obtained, an expression vector comprising the DNA and a host transformed with the vector can be obtained according to conventional methods. In addition, scFv can be obtained according to conventional methods using the host.

As above, these antibody fragments can be produced from the host by obtaining and expressing their genes. Herein, an "antibody" encompasses such antibody fragments.

Antibodies bound to various molecules, such as polyethylene glycol (PEG), may also be used as modified antibodies. Herein, an "antibody" encompasses such modified antibodies. These modified antibodies can be obtained by chemically modifying the obtained antibodies. Such methods are already established in the art.

Antibodies produced and expressed as above can be isolated from the inside or outside of the cells or from the hosts, and then purified to homogeneity. The antibodies for use in the present invention can be isolated and/or purified using affinity chromatography. Columns to be used for the affinity chromatography include, for example, protein A columns and protein G columns. Carriers used for the protein A columns include, for example, HyperD, POROS, Sepharose FF and such. In addition to the above, other methods used for the isolation and/or purification of common proteins may be used, and are not limited in any way.

For example, the antibodies used for the present invention may be isolated and/or purified by appropriately selecting and combining chromatographies in addition to affinity chromatography, filters, ultrafiltration, salting-out, dialysis, and such. Chromatographies include, for example, ion-exchange chromatography, hydrophobic chromatography, gel filtration, and such. These chromatographies can be applied to high performance liquid chromatography (HPLC). Alternatively, reverse phase HPLC may be used.

The concentration of the antibodies obtained as above can be determined by absorbance measurement, ELISA, or such. Specifically, absorbance is determined by appropriately diluting the antibody solution with PBS(−), measuring absorbance at 280 nm, and calculating the concentration (1.35 OD=1 mg/ml). Alternatively, when using ELISA, the measurement can be performed as follows: Specifically, 100 μl of goat anti-human IgG (TAG) diluted to 1 μg/ml with 0.1 M bicarbonate buffer (pH 9.6) is added to a 96-well plate (Nunc) and incubated overnight at 4° C. to immobilize the antibody. After blocking, 100 μl of an appropriately diluted antibody of the present invention or an appropriately diluted sample comprising the antibody, and human IgG (CAPPEL) are added as a standard, and incubated for one hour at room temperature.

After washing, 100 μl of 5,000× diluted alkaline phosphatase-labeled anti-human IgG (BIO SOURCE) is added and incubated for one hour at room temperature. After another wash, substrate solution is added and incubated, and the absorbance at 405 nm is measured using a Microplate Reader Model 3550 (Bio-Rad) to calculate the concentration of the antibody of interest.

The IL-6 variants used in the present invention are substances with the activity of binding to an IL-6 receptor and which do not transmit IL-6 biological activity. That is, the IL-6 variants compete with IL-6 to bind to IL-6 receptors, but fail to transmit IL-6 biological activity, and hence the block IL-6-mediated signal transduction.

The IL-6 variants are produced by introducing mutation(s) by substituting amino acid residues in the amino acid sequence of IL-6. The origin of IL-6 used as the base of the IL-6 variants is not limited, but is preferably human IL-6 in consideration of antigenicity and such.

More specifically, amino acid substitutions are performed by predicting the secondary structure of the IL-6 amino acid sequence using known molecular modeling programs (e.g., WHATIF; Vriend et al., J. Mol. Graphics (1990) 8, 52-56), and further assessing the influence of the substituted amino acid residue(s) on the whole molecule. After determining the appropriate amino acid residue to be substituted, commonly performed PCR methods are carried out using a nucleotide sequence encoding a human IL-6 gene as a template, and mutations are introduced to cause amino acids substitutions, and thus genes encoding IL-6 variants are obtained. If needed, this gene is inserted into an appropriate expression vector, and the IL-6 variant can be obtained by applying the aforementioned methods for expression, production, and purification of recombinant antibodies.

Specific examples of the IL-6 variants are disclosed in Brakenhoff et al., J. Biol. Chem. (1994) 269, 86-93, Savino et al., EMBO J. (1994) 13, 1357-1367, WO 96/18648, and WO 96/17869.

The partial peptides of IL-6 and of the IL-6 receptor to be used in the present invention are substances with the activity of binding to the IL-6 receptor and to IL-6, respectively, and which do not transmit IL-6 biological activity. Namely, by binding to and capturing an IL-6 receptor or IL-6, the IL-6 partial peptides or IL-6 receptor partial peptides can specifically inhibit IL-6 from binding to the IL-6 receptor. As a result, the biological activity of IL-6 is not transmitted, and IL-6-mediated signal transduction is blocked.

The partial peptides of IL-6 or IL-6 receptor are peptides that comprise part or all of the amino acid sequence of the region of the IL-6 or IL-6 receptor amino acid sequence that is involved in the binding between the IL-6 and IL-6 receptor. Such peptides usually comprise ten to 80, preferably 20 to 50, more preferably 20 to 40 amino acid residues.

The IL-6 partial peptides or IL-6 receptor partial peptides can be produced according to generally known methods, for example, genetic engineering techniques or peptide synthesis methods, by specifying the region of the IL-6 or IL-6 receptor amino acid sequence that is involved in the binding between the IL-6 and IL-6 receptor, and using a portion or entirety of the amino acid sequence of the specified region.

When preparing an IL-6 partial peptide or IL-6 receptor partial peptide using genetic engineering methods, a DNA sequence encoding the desired peptide is inserted into an expression vector, and then the peptide can be obtained by applying the aforementioned methods for expressing, producing, and purifying recombinant antibodies.

When producing an IL-6 partial peptide or IL-6 receptor partial peptide by using peptide synthesis methods, generally used peptide synthesis methods, for example, solid phase synthesis methods or liquid phase synthesis methods, may be used.

Specifically, the peptides can be synthesized according to the method described in "Continuation of Development of Pharmaceuticals, Vol. 14, Peptide Synthesis (in Japanese) (ed. Haruaki Yajima, 1991, Hirokawa Shoten)". As a solid phase synthesis method, for example, the following method can be employed: the amino acid corresponding to the C terminus of the peptide to be synthesized is bound to a support that is insoluble in organic solvents, then the peptide strand is elongated by alternately repeating (1) the reaction of condensing amino acids, whose α-amino groups and branch chain functional groups are protected with appropriate protecting groups, one at a time in a C- to N-terminal direction; and (2) the reaction of removing the protecting groups from the α-amino groups of the resin-bound amino acids or peptides. Solid phase peptide synthesis is broadly classified into the Boc method and the Fmoc method, depending on the type of protecting groups used.

After synthesizing a protein of interest as above, deprotection reactions are carried out, then the peptide strand is cleaved from its support. For the cleavage reaction of the peptide strand, hydrogen fluoride or trifluoromethane sulfonic acid are generally used for the Boc method, and TFA is generally used for the Fmoc method. In the Boc method, for example, the above-mentioned protected peptide resin is treated with hydrogen fluoride in the presence of anisole. Then, the peptide is recovered by removing the protecting groups and cleaving the peptide from its support. By freeze-drying the recovered peptide, a crude peptide can be obtained. In the Fmoc method, on the other hand, the deprotection reaction and the reaction to cleave the peptide strand from the support can be performed in TFA using a method similar to those described above, for example.

Obtained crude peptides can be separated and/or purified by applying HPLC. Elution may be performed under optimum conditions using a water-acetonitrile solvent system, which is generally used for protein purification. The fractions corresponding to the peaks of the obtained chromatographic profile are collected and freeze-dried. Thus, purified peptide fractions are identified by molecular weight analysis via mass spectrum analysis, amino acid composition analysis, amino acid sequence analysis, or such.

Specific examples of IL-6 partial peptides and IL-6 receptor partial peptides are disclosed in JP-A Hei 2-188600, JP-A Hei 7-324097, JP-A Hei 8-311098, and U.S. Pat. No. 5,210,075.

The antibodies used in the present invention may also be conjugated antibodies that are bound to various molecules, such as polyethylene glycol (PEG), radioactive substances, and toxins. Such conjugated antibodies can be obtained by chemically modifying the obtained antibodies. Methods for modifying antibodies are already established in the art. The "antibodies" of the present invention encompass these conjugated antibodies.

The agents of the present invention for suppressing cytotoxic T cell induction, which comprise IL-6 inhibitors as active ingredients, can be used to suppress post-transplantation rejection.

The present invention also provides agents for suppressing rejection in heart transplantation, which comprise IL-6 inhibitors as active ingredients.

The types of rejection suppressed by the suppressing agents of the present invention are not particularly limited, but are preferably acute rejections, which become problematic in actual transplantation medicine. These rejections are pathological states in which allografts are recognized as foreign antigens due to differences in the major histocompatibility complex (MHC) that determines histocompatibility, and are thus attacked through activation of the recipient's cytotoxic T cells and helper T cells. Rejections are generally developed within three months of transplantation. However, rejections can also be recognized as cell infiltrations into the allograft tissue, three months or more after transplantation.

Herein, "suppressing rejection after transplantation" means improving viability of a transplanted organ by suppressing damage to it.

The types of organ transplantations for which the suppressing agents of the present invention can be used are not particularly limited, and preferred organs for the organ transplantations in the present invention include parenchymal organs, such as hearts, livers, kidneys, pancreas, lungs, and small intestines. The present invention can also be applied to transplantation of tissues such as cardiac valves, vessels, skin, bones, and corneas.

The suppression of a post-transplantation rejection can be confirmed by using a method described in the Examples to determine CTL activity in the subjects following administration of a suppressing agent of the present invention. When a recipient's CTL activity towards a donor's HLA antigen is continuously reduced upon administration of a suppressing agent of the present invention, the agent can be said to suppress transplant failures. When organ viability is improved as a result, the agent can also be said to "suppress transplant failures" in the organ transplantation. The survival of each organ can be assessed by examining whether organ function becomes normal after transplantation.

In the present invention, the activity of IL-6 inhibitors in inhibiting the transduction of IL-6 signals can be evaluated by conventional methods. Specifically, IL-6 is added to cultures of IL-6-dependent human myeloma cell lines (S6B45 and KPMM2), human Lennert T lymphoma cell line KT3, or IL-6-dependent cell line MH60.BSF2; and the ^{3}H-thymidine uptake by the IL-6-dependent cells is measured in the presence of an IL-6 inhibitor. Alternatively, IL-6 receptor-expressing U266 cells are cultured, and ^{125}I-labeled IL-6 and an IL-6 inhibitor are added to the culture at the same time; and then ^{125}I-labeled IL-6 bound to the IL-6 receptor-expressing cells is quantified. In addition to the IL-6 inhibitor group, a negative control group that does not contain an IL-6 inhibitor is included in the assay system described above. The activity of the IL-6 inhibitor to inhibit IL-6 can be evaluated by comparing the results of both groups.

Subjects to be administered with the suppressing agents of the present invention are mammals. The mammals are preferably humans.

The suppressing agents of the present invention can be administered as pharmaceuticals, and may be administered

systemically or locally via oral or parenteral administration. For example, intravenous injections such as drip infusions, intramuscular injections, intraperitoneal injections, subcutaneous injections, suppositories, enemas, oral enteric tablets, or the like can be selected. Appropriate administration methods can be selected depending on a patient's age and symptoms. The effective dose per administration is selected from the range of 0.01 to 100 mg/kg body weight. Alternatively, the dose may be selected from the range of 1 to 1000 mg/patient, preferably from the range of 5 to 50 mg/patient. A preferred dose and administration method are as follows: For example, when an anti-IL-6 receptor antibody is used, the effective dose is an amount such that free antibody is present in the blood. Specifically, a dose of 0.5 to 40 mg/kg body weight/month (four weeks), preferably 1 to 20 mg/kg body weight/month is administered via an intravenous injection such as a drip infusion, subcutaneous injection or such, once to several times a month, for example, twice a week, once a week, once every two weeks, or once every four weeks. The administration schedule may be adjusted by, for example, extending the administration interval of twice a week or once a week to once every two weeks, once every three weeks, or once every four weeks, while monitoring the condition after transplantation and changes in the blood test values.

In the present invention, the suppressing agents may contain pharmaceutically acceptable carriers, such as preservatives and stabilizers. "Pharmaceutically acceptable carriers" refer to materials that can be co-administered with an above-described agent; and may or may not themselves produce the above-described effect of suppressing cytotoxic T cell induction. Alternatively, the carriers may be materials that do not have the effect of suppressing cytotoxic T cell induction, but that produce an additive or synergistic stabilizing effect when used in combination with an IL-6 inhibitor.

Such pharmaceutically acceptable materials include, for example, sterile water, physiological saline, stabilizers, excipients, buffers, preservatives, detergents, chelating agents (EDTA and such), and binders.

In the present invention, detergents include non-ionic detergents, and typical examples of such include sorbitan fatty acid esters such as sorbitan monocaprylate, sorbitan monolaurate, and sorbitan monopalmitate; glycerin fatty acid esters such as glycerin monocaprylate, glycerin monomyristate and glycerin monostearate; polyglycerin fatty acid esters such as decaglyceryl monostearate, decaglyceryl distearate, and decaglyceryl monolinoleate; polyoxyethylene sorbitan fatty acid esters such as polyoxyethylene sorbitan monolaurate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene sorbitan trioleate, and polyoxyethylene sorbitan tristearate; polyoxyethylene sorbit fatty acid esters such as polyoxyethylene sorbit tetrastearate and polyoxyethylene sorbit tetraoleate; polyoxyethylene glycerin fatty acid esters such as polyoxyethylene glyceryl monostearate; polyethylene glycol fatty acid esters such as polyethylene glycol distearate; polyoxyethylene alkyl ethers such as polyoxyethylene lauryl ether; polyoxyethylene polyoxypropylene alkyl ethers such as polyoxyethylene polyoxypropylene glycol, polyoxyethylene polyoxypropylene propyl ether, and polyoxyethylene polyoxypropylene cetyl ether; polyoxyethylene alkyl phenyl ethers such as polyoxyethylene nonylphenyl ether; polyoxyethylene hardened castor oils such as polyoxyethylene castor oil and polyoxyethylene hardened castor oil (polyoxyethylene hydrogenated castor oil); polyoxyethylene beeswax derivatives such as polyoxyethylene sorbit beeswax; polyoxyethylene lanolin derivatives such as polyoxyethylene lanolin; and polyoxyethylene fatty acid amides and such with an HLB of six to 18, such as polyoxyethylene stearic acid amide.

Detergents also include anionic detergents, and typical examples of such include, for example, alkylsulfates having an alkyl group with ten to 18 carbon atoms, such as sodium cetylsulfate, sodium laurylsulfate, and sodium oleylsulfate; polyoxyethylene alkyl ether sulfates in which the alkyl group has ten to 18 carbon atoms and the average molar number of added ethylene oxide is 2 to 4, such as sodium polyoxyethylene lauryl sulfate; alkyl sulfosuccinate ester salts having an alkyl group with eight to 18 carbon atoms, such as sodium lauryl sulfosuccinate ester; natural detergents, for example, lecithin; glycerophospholipids; sphingo-phospholipids such as sphingomyelin; and sucrose fatty acid esters in which the fatty acids have 12 to 18 carbon atoms.

One, two or more of the detergents described above can be combined and added to the agents of the present invention. Detergents that are preferably used in the preparations of the present invention include polyoxyethylene sorbitan fatty acid esters, such as polysorbates 20, 40, 60, and 80. Polysorbates 20 and 80 are particularly preferred. Polyoxyethylene polyoxypropylene glycols, such as poloxamer (Pluronic F-68® and such), are also preferred.

The amount of detergent added varies depending on the type of detergent used. When polysorbate 20 or 80 is used, the amount is in general in the range of 0.001 to 100 mg/ml, preferably in the range of 0.003 to 50 mg/ml, more preferably in the range of 0.005 to 2 mg/ml.

In the present invention, buffers include phosphate, citrate buffer, acetic acid, malic acid, tartaric acid, succinic acid, lactic acid, potassium phosphate, gluconic acid, capric acid, deoxycholic acid, salicylic acid, triethanolamine, fumaric acid, and other organic acids; and carbonic acid buffer, Tris buffer, histidine buffer, and imidazole buffer.

Liquid preparations may be formulated by dissolving the agents in aqueous buffers known in the field of liquid preparations. The buffer concentration is in general in the range of 1 to 500 mM, preferably in the range of 5 to 100 mM, more preferably in the range of 10 to 20 mM.

The agents of the present invention may also comprise other low-molecular-weight polypeptides; proteins such as serum albumin, gelatin, and immunoglobulin; amino acids; sugars and carbohydrates such as polysaccharides and monosaccharides, sugar alcohols, and such.

Herein, amino acids include basic amino acids, for example, arginine, lysine, histidine, and ornithine, and inorganic salts of these amino acids (preferably hydrochloride salts, and phosphate salts, namely phosphate amino acids). When free amino acids are used, the pH is adjusted to a preferred value by adding appropriate physiologically acceptable buffering substances, for example, inorganic acids, and in particular hydrochloric acid, phosphoric acid, sulfuric acid, acetic acid, and formic acid, and salts thereof. In this case, the use of phosphate is particularly beneficial because it gives quite stable freeze-dried products. Phosphate is particularly advantageous when preparations do not substantially contain organic acids, such as malic acid, tartaric acid, citric acid, succinic acid, and fumaric acid, or do not contain corresponding anions (malate ion, tartrate ion, citrate ion, succinate ion, fumarate ion, and such). Preferred amino acids are arginine, lysine, histidine, and ornithine. Acidic amino acids can also be used, for example, glutamic acid and aspartic acid, and salts thereof (preferably sodium salts); neutral amino acids, for example, isoleucine, leucine, glycine, serine, threonine, valine, methionine, cysteine, and alanine; and aromatic amino acids, for example, phenylalanine, tyrosine, tryptophan, and its derivative, N-acetyl tryptophan.

Herein, sugars and carbohydrates such as polysaccharides and monosaccharides include, for example, dextran, glucose, fructose, lactose, xylose, mannose, maltose, sucrose, trehalose, and raffinose.

Herein, sugar alcohols include, for example, mannitol, sorbitol, and inositol.

When the agents of the present invention are prepared as aqueous solutions for injection, the agents may be mixed with, for example, physiological saline, and/or isotonic solution containing glucose or other auxiliary agents (such as D-sorbitol, D-mannose, D-mannitol, and sodium chloride). The aqueous solutions may be used in combination with appropriate solubilizing agents such as alcohols (ethanol and such), polyalcohols (propylene glycol, PEG and such), or non-ionic detergents (polysorbate 80 and HCO-50).

The agents may further comprise, if required, diluents, solubilizers, pH adjusters, soothing agents, sulfur-containing reducing agents, antioxidants, and such.

Herein, the sulfur-containing reducing agents include, for example, compounds comprising sulfhydryl groups, such as N-acetylcysteine, N-acetylhomocysteine, thioctic acid, thiodiglycol, thioethanolamine, thioglycerol, thiosorbitol, thioglycolic acid and salts thereof, sodium thiosulfate, glutathione, and thioalkanoic acids having one to seven carbon atoms.

Moreover, the antioxidants in the present invention include, for example, erythorbic acid, dibutylhydroxy toluene, butylhydroxy anisole, a-tocopherol, tocopherol acetate, L-ascorbic acid and salts thereof, L-ascorbic acid palmitate, L-ascorbic acid stearate, sodium hydrogen sulfite, sodium sulfite, triamyl galiate, propyl gallate, and chelating agents such as disodium ethylenediamine tetraacetate (EDTA), sodium pyrophosphate, and sodium metaphosphate.

If required, the agents may be encapsulated in microcapsules (microcapsules of hydroxymethylcellulose, gelatin, poly[methylmethacrylic acid] or such) or prepared as colloidal drug delivery systems (liposome, albumin microspheres, microemulsion, nano-particles, nano-capsules, and such) (see "Remington's Pharmaceutical Science 16th edition", Oslo Ed., 1980, and the like). Furthermore, methods for preparing agents as sustained-release agents are also known, and are applicable to the present invention (Langer et al., J. Biomed. Mater. Res. 1981, 15: 167-277; Langer, Chem. Tech. 1982, 12: 98-105; U.S. Pat. No. 3,773,919; European Patent Application No. (EP) 58, 481; Sidman et al., Biopolymers 1983, 22: 547-556; and EP 133,988).

Pharmaceutically acceptable carriers used are appropriately selected from those described above or combined depending on the type of dosage form, but are not limited thereto.

The present invention relates to methods for suppressing rejection after transplantation, which comprise the step of administering IL-6 inhibitors to subjects.

The present invention also relates to methods for suppressing rejection in heart transplantation, which comprise the step of administering IL-6 inhibitors to subjects.

The types of rejections suppressed by the methods of the present invention are not particularly limited, and acute rejections are preferable. The types of organ transplantations for which the suppressing agents of the present invention can be used are not particularly limited. Preferred organs for the organ transplantations in the present invention include parenchymal organs, such as hearts, livers, kidneys, pancreas, lungs, and small intestines. The present invention can also be applied to transplantation of tissues such as cardiac valves, vessels, skin, bones, and corneas.

Herein, the "subject" refers to the organisms or organism body parts to be administered with an IL-6 inhibitor of the present invention. The organisms include animals (for example, human, domestic animal species, and wild animals) but are not particularly limited. The "organism body parts" are not particularly limited.

Herein, "administration" includes oral and parenteral administration. Oral administration includes, for example, administration of oral agents. Such oral agents include, for example, granules, powders, tablets, capsules, solutions, emulsions, and suspensions.

Parenteral administration includes, for example, administration of injections. Such injections include, for example, subcutaneous injections, intramuscular injection s, and intraperitoneal injection. Meanwhile, the effects of the methods of the present invention can be achieved by introducing genes comprising oligonucleotides to be administered to living bodies using gene therapy techniques. Alternatively, the agents of the present invention may be administered locally to intended areas of treatment. For example, the agents can be administered by local injection during surgery, use of catheters, or targeted gene delivery of DNAs encoding peptides of the present invention.

The suppressing agents of the present invention may be administered to subjects prior to organ transplantation, at the time of organ transplantation, or after organ transplantation. Further, the suppressing agents may be administered once or repeatedly.

Alternatively, when administered to an excised or delivered part of an organism, the suppressing agents of the present invention may be "contacted" with the organism part.

In the present invention, "contacting" is performed according to the condition of the organism. Examples include spraying the suppressing agents of the present invention over the organism parts, and adding the suppressing agents of the present invention to crushed organism parts, but are not limited thereto. When the organism part is cultured cells, the above-mentioned "contact" can be achieved by adding the suppressing agents of the present invention to culture medium of these cells, or by introducing DNAs comprising oligonucleotides of the present invention into cells that constitute the organism part.

When conducting the methods of the present invention, the agents of the present invention may be administered as parts of pharmaceutical compositions in combination with at least one known chemotherapeutant. Alternatively, the agents of the present invention may be administered simultaneously with at least one known immunosuppressant. In one embodiment, the known chemotherapeutants and the suppressing agents of the present invention may be administered virtually simultaneously.

The agents for suppressing the induction of cytotoxic T cells of the present invention may be administered to sites of organ transplantation after the organ have been transplanted, or may be administered to targets at the same time as the organ. Alternatively, the agents may be added to the organ in vitro, prior to transplantation.

All prior-art documents cited herein are incorporated herein by reference.

EXAMPLES

Hereinbelow, the present invention will be specifically described with reference to the Examples, but it is not to be construed as being limited thereto.

Example 1

Analysis of the Effect of Anti-IL-6 Receptor Antibody Administration on CTL Activity Against Cells of the Mastocytoma Line P815

First, 3×10^7 cells of the mastocytoma line P815 (immunizing cells) were administered intraperitoneally into C57BL/6 mice. After 13 days, spleens were isolated from the mice and the splenic cells (effector cells) were co-cultured with ^{51}Cr-labeled P815 cells (target cells) at various effector cell/target cell (E/T) ratios to assay the CTL activity against ^{51}Cr-labeled P815.

CTL activity was assayed by determining the cell lysis rate for each cell sample. Cell lysis rate is expressed as follows:

[(the amount of ^{51}Cr released in an experiment−the amount of spontaneously released ^{51}Cr)/(the total amount of released ^{51}Cr−the amount of spontaneously released ^{51}Cr)]×100.

Next, 2000 mg and 500 mg of an anti-IL-6 receptor antibody (MR16-1) were intraperitoneally administered four days and one day before isoantigen administration, respectively. Then, four and nine days after isoantigen administration, 500 mg of the antibody was administered. CTL activity was determined by the methods described above. Meanwhile, under the same treatment conditions, control mice were injected with rat IgG as a control antibody, instead of the anti-IL-6 receptor antibody. CTL activity was determined by the same method as described above.

The results obtained in the assays described above showed that CTL activity against the alloantigen (mastocytoma line P815) was statistically significantly reduced in mice treated with the anti-IL-6 receptor antibody as compared to mice not treated with any antibodies and mice treated with the control antibody (FIGS. **1** and **2**). ($p<0.01$) When P815 cells were introduced into IL-6-deficient C57BL/6 mice, the level of in-vivo CTL induction was found to be significantly reduced (data not shown).

Example 2

Analysis of the Effect of Anti-IL-6 Receptor Antibody Administration on CTL Activity Against Cells of the EL4 Lymphoma Cell Line

By the same procedure as described above, 3×10^7 cells of the EL4 lymphoma cell line (immunizing cells) were administered intraperitoneally into BALB/c mice. After 13 days, spleens were isolated from the mice and the splenic cells (effector cells) were co-cultured with ^{51}Cr-labeled EL4 cells (target cells) at various effector cell/target cell (E/T) ratios. CTL activity against ^{51}Cr-labeled EL4 was assayed using the same method as described in Example 1. Next, using the same procedure as described in Example 1, 2000 mg and 500 mg of the anti-IL-6 receptor antibody were intraperitoneally administered four days and one day before isoantigen administration, respectively. Then, four and nine days after isoantigen administration, 500 mg of the antibody was administered. CTL activity was determined by the method described above. In addition, under the same treatment conditions, control mice were injected with rat IgG as a control antibody, instead of the anti-IL-6 receptor antibody. CTL activity was determined using the methods described above.

The results obtained in the assays described above showed that CTL activity against the alloantigen (EL4 lymphoma line) was statistically significantly reduced in mice treated with the anti-IL-6 receptor antibody, as compared with mice not treated with any antibodies and mice treated with the control antibody (FIGS. **3** and **4**). ($p<0.01$)

The results described above showed that the anti-IL-6 receptor antibody had functions to suppress cytotoxic T cell induction.

Example 3

Assessment of the Effect of Anti-IL-6 Receptor Antibody Administration in a Mouse Model for Acute Heart Transplant Rejection

The effect of administering an anti-IL-6 receptor antibody (MR16-1) on acute rejection after transplantation was assessed using a mouse model for heart transplantation. The mice used in the experiments were purchased from Japan SLC Inc. and bred at the Division of Laboratory Animal Research, Department of Life Science, Research Center for Human and Environmental Sciences, Shinshu University, according to the center's animal experiment protocol. A mouse model for heterotopic heart transplantation using BALB/c mouse donors and C57BL/6 mouse recipients, which are allogenic, was prepared by microsurgery using the procedure described below. The donors and recipients used were four- to eight-week-old male mice.

Both donor and recipient mice were anesthetized by intraperitoneally injecting pentobarbital sodium (Nenbutal(R)) at a dose of 70 mg/kg. The heart to be transplanted was isolated after ligating vessels other than ascending aorta and pulmonary artery to be used for anastomosis. The isolated heart graft was preserved in cold physiological saline containing 7.5% heparin on ice. The recipient was laparotomized in the midline and the intestines were flipped over to expose the abdominal aorta and inferior vena cava. After blood flow was stopped using microclips for microvessels, an about 1-mm incision was created on each surface for anastomotic sites. The aorta and the pulmonary artery of the transplanted heart were anastomosed to the abdominal aorta and the inferior vena cava of the recipient, respectively, by continuous suture using 10-0 nylon suture. The microclips were unfastened gradually to resume the blood flow. The transplanted heart was confirmed to resume beating. After confirming hemorrhage arrest, the abdominal wall and the skin were sutured to close the abdomen.

Each surgery took about 45 minutes. The success rate was 95% or greater.

The treated group were intraperitoneally injected with the anti-IL-6 receptor antibody at a dose of 2 mg/mouse/administration immediately, three days, and six days after transplantation. Beating of the transplanted heart was confirmed every day by abdominal palpation. When the heart completely stopped beating, the heart was concluded to have been rejected. At that time, the recipient was anesthetized and laparotomized to confirm by visual inspection that the heart had stopped beating. Time until rejection was defined as the survival period of the cardiac allograft. The periods were compared with each other.

The results showed that administration of the anti-IL-6 receptor antibody suppressed acute rejection of cardiac allografts and statistically significantly prolonged the allograft survival (FIG. **5**). ($p=0.0001$)

Untreated group: 7, 7, 7, 7, 7, 7, 8, 9, 9, 9, and 10 (days). (11 cases, Median survival time: seven days).

Anti-IL-6 receptor antibody-treated group: 11, 13, 16, 17, 20, and 21 (days). (six cases, Median survival time: 16.5 days).

Furthermore, five days after transplantation, the cardiac allografts were isolated from the recipients for histological examination.

Pathological tissue sections were prepared from frozen tissue samples of the cardiac allografts five days after transplantation, and these were stained with hematoxylin-eosin. Diffuse infiltration of inflammatory cells and myocardial necrosis were found in the untreated group. In the anti-IL-6 receptor antibody-treated group, the infiltration of inflammatory cells was mild and cardiomyocytes were relatively preserved (FIG. **6**).

The results of histological analysis were scored according to the five step criteria of "0: no rejection" to "4: severe rejection" (Rodriguez, E. R. The pathology of heart transplant biopsy specimens: revisiting the 1990 ISHLT working formulation. J Heart Lung Transplant. (2003) 22, 3-15% Billingham, M. E. et al., A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J Heart Transplant. (1990) 9, 587-93). When the rejection score for the anti-IL-6 receptor antibody-treated group was compared with that of the untreated group, significant suppression was revealed in the treated group (FIG. **7**). (p=0.0006)

The above results showed that administration of an anti-IL-6 receptor antibody to recipients suppressed acute rejection of cardiac allografts and statistically significantly prolonged the allograft survival in the mouse model for heart transplantation.

INDUSTRIAL APPLICABILITY

The present invention demonstrated that cytotoxic T cells induction could be suppressed by administering an anti-IL-6 receptor antibody.

The invention also demonstrated that immunorejections after transplantation could be suppressed by administering the anti-IL-6 receptor antibody.

The invention claimed is:

1. A method for suppressing acute rejection of a heart transplant in a subject, the method comprising administering to the subject an interleukin-6 (IL-6) biological activity inhibitor that is an antibody that recognizes an IL-6 receptor, thereby suppressing acute rejection of the heart transplant in the subject.

2. The method of claim **1**, wherein the antibody is a monoclonal antibody.

3. The method of claim **1**, wherein the antibody recognizes a human IL-6 receptor.

4. The method of claim **1**, wherein the antibody is a recombinant antibody.

5. The method of claim **1**, wherein the antibody is a chimeric antibody, humanized antibody, or human antibody.

6. The method of claim **1**, wherein the antibody is a scFv.

7. The method of claim **1**, wherein the inhibitor is administered to the subject repeatedly.

8. The method of claim **1**, wherein the inhibitor is administered prior to transplantation of the heart into the subject.

9. The method of claim **1**, wherein the inhibitor is administered at the time of transplantation of the heart into the subject.

10. The method of claim **1**, wherein the inhibitor is administered after transplantation of the heart into the subject.

11. The method of claim **1**, wherein the inhibitor is administered immediately after transplantation of the heart into the subject.

12. The method of claim **1**, wherein the inhibitor is administered three days after transplantation of the heart into the subject.

13. The method of claim **1**, wherein the inhibitor is administered six days after transplantation of the heart into the subject.

14. The method of claim **1**, wherein the method further comprises confirming that acute rejection of the transplanted heart is suppressed.

15. The method of claim **1**, wherein the method further comprises assaying cytotoxic T cell activity in the subject following administration of the inhibitor.

16. The method of claim **1**, wherein the subject is a human subject.

17. The method of claim **1**, wherein the antibody is administered in a dose of 0.01 to 100 mg/kg per administration.

18. The method of claim **1**, wherein the antibody is administered in a dose of 1 to 1000 mg/patient.

19. The method of claim **1**, wherein the antibody is administered in a dose of 0.5 to 40 mg/kg body weight/month.

20. The method of claim **1**, wherein the inhibitor is administered to the subject once.

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